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# Human Papillomavirus DNA Detection in Menstrual Blood from Patients with Cervical Intraepithelial Neoplasia and Condyloma Acuminatum▽

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**The Papanicolaou test generates pain and embarrassment, and cytology screening has limited sensitivity for detection of cervical neoplasia. These factors urge the use of another screening test that can overcome these limitations. We explore a completely noninvasive method using detection of human papillomavirus (HPV) DNA in women's menstrual blood (MB). The participants were divided into 3 cohorts: (i) 235 patients with cervical intraepithelial neoplasia 3 (CIN 3) ($n = 48$), CIN 2 ($n = 60$), CIN 1 ($n = 58$), or condyloma acuminatum (CAC) ($n = 69$) before treatment or remission; (ii) from the first cohort of patients, 108 CIN 3 or CIN 2 patients after treatment and 62 CIN 1 or CAC patients after remission; and (iii) 323 apparently normal subjects (ANS) without any cervical disease. The HPV genotypes of the infected patients were confirmed by direct sequencing. Quantitative real-time PCR (QRT-PCR) was used to measure the MB HPV16 load for 15 infected patients. Results showed that the sensitivity, specificity, and positive and negative predictive values for detection of MB HPV DNA in samples from patients with CIN or CAC were 82.8%, 93.1%, 90.0%, and 87.9%, respectively. Moreover, MB HPV DNA was found in samples from 22.2% of CIN 3 or CIN 2 patients after treatment, 0.0% of CIN 1 or CAC patients after remission, and 8.1% of ANS, 4 of whom were found to have CIN 1 or CAC. Furthermore, QRT-PCR showed that the normalized MB HPV16 DNA copy numbers in samples from patients with CIN 1 to CIN 3 were significantly increased. These preliminary results suggested that MB HPV DNA is a potential noninvasive marker for these premalignant cervical diseases.**

Cervical cancer (CC) is the second most common malignancy and cause of cancer-related death in women worldwide (17). The well-defined premalignant phase of this cancer has contributed to the success of the cytology-based Papanicolaou (Pap) screening test; the incidence of CC in resource-rich countries has been dramatically reduced by the use of this test (11). However, the Pap test has several limitations: (i) the collection of cervical cells can create discomfort and embarrassment (7); (ii) evaluation of test results in cytology screening involves subjective assessments with high susceptibility to intraindividual and interindividual variability (11); (iii) the test exhibits low (around 51%) sensitivity for detection of high-grade (HG) cervical intraepithelial neoplasia (HGCIN) (11); and (iv) cytology-trained personnel and special equipment with excellent quality control are needed, which involves a huge amount of resources (3). Therefore, there is a need for another cervical screening test that can overcome these limitations. Recent studies have shown that the human papillomavirus (HPV) DNA test is more sensitive than the Pap test in detecting HGCIN (11–13). However, the sample collection method, using a cytobrush, is still invasive and unpleasant, which would affect a woman's decision whether to take the Pap test. We hypothesized that menstrual blood (MB) collected in sanitary napkins would contain HPV-infected cervical cells from patients with CIN or condyloma acuminatum (CAC), as HPV is known to be the etiological agent for both conditions (10). In this study, we explored the possibility of detecting and typing HPV DNA in MB collected in sanitary napkins from patients with CIN or CAC. As a high HPV16 DNA load has been reported to be associated with a higher grade of CIN or a higher risk of development of HGCIN during follow-up (5, 19), it would be interesting to explore whether HPV16 DNA loads can be measured using quantitative real-time PCR (QRT-PCR) with MB specimens. The information obtained would be important for enhancing understanding of the diagnostic and prognostic potential of MB HPV DNA

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for patients with CIN and CAC, and possibly for determining whether MB HPV DNA can be used as the basis for a noninvasive screening test for detection of these premalignant cervical diseases (PCD).

## MATERIALS AND METHODS

**Study subjects.** Two hundred and thirty-five patients (age range, 17 to 54 years; mean age, 37.3 years) with histological diagnosis of CIN 3 ($n = 48$), CIN 2 ($n = 60$), CIN 1 ($n = 58$), or CAC ($n = 69$) before treatment or remission were recruited in the Department of Obstetrics and Gynaecology, Queen Elizabeth Hospital, Hong Kong Special Administrative Region (HKSAR), China, from 2007 to 2009. CIN is the designation for the premalignant lesion category, members of which are divided into HGCIN (CIN 3 and CIN 2) and low-grade CIN (LGCIN) (CIN 1), whereas CAC is the designation for the benign lesion in the cervix (10). Within this cohort, 108 patients with CIN 3 or CIN 2 had undergone treatment that included a loop electrosurgical excision procedure (LEEP); 62 patients with CIN 1 or CAC with complete remission as proven by the Pap test were also recruited. Finally, 323 sexually active, apparently normal subjects (ANS) (age range, 24 to 50 years; mean age, 36.4 years) who did not have any cervical disease before the study were recruited as a control group. All participants were subjected to a short interview, including a brief introduction to this study, and to questions concerning their medical, gynecological, and sexual histories. ANS with positive and negative MB HPV DNA test results were all referred for a Pap test and followed by colposcopy with histological confirmation if the Pap test result was found to show atypical squamous cells of undetermined significance or indicating the presence of CIN. The study was approved by the Clinical Research Ethics Committee, Queen Elizabeth Hospital, HKSAR, China. Written consent was obtained from all participants.

**MB HPV DNA detection and genotyping.** MB collected in a sanitary napkin was put inside a ziplock plastic bag, which was sent to the laboratory by mail or by hand delivery. A small (1.5 cm by 1.5 cm by 5 mm) piece of sanitary napkin was cut out using sterile scissors. Genomic DNA was extracted from a small piece of sanitary napkin with MB by the use of a commercial QIAamp DNA Mini kit (catalog no. 51306; Qiagen, Hilden, Germany) according to the dried blood spot protocol. HPV DNA detection was performed by the use of two rounds of 50 cycles of PCR using the same set of My11 and My09 degenerate primers. Those primers were targeted at the conserved L1 region of the HPV genome, which allowed the detection of a broad range of HPV types (9). First-round PCR was performed using a reaction volume of 20 μl, and 100 ng of DNA was used for each reaction. For the second-round PCR, 1 μl of the first-round PCR product was used in a reaction volume of 20 μl. β-Globin DNA detection was performed for all samples as a housekeeping control using another pair of established primers (15). Reactions were performed in duplicate, and HPV types were confirmed by direct sequencing using the My11 primer. The sequencing products were analyzed using an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, CA), and sequence homology was examined by the use of the NCBI BLAST search program.

**MB HPV16 DNA measurement using QRT-PCR.** To measure MB HPV16 DNA copy numbers, primers and a TaqMan minor grove binder (MGB) probe, labeled with a 6-carboxyfluorescein (FAM) reporter dye at the 5′ end and a nonfluorescent quencher at the 3′ end, were designed at the E6 open reading frame (forward primers, 5′-CACCAAAAGAGAACTGCAATGTTT-3′; reverse primers, 5′-TTTGCAGCTCTGTGCATAACTGT-3′; probe, 5′FAM-ACCCAC AGGAGCGAC-3′).

Specificity of the HPV16 DNA primers and probes was validated by quantifying MB HPV16 DNA from patients with HPV16 infections, patients with HPV other than type 16 infections, and normal subjects without HPV infection. Results were positive for all HPV16 DNA-positive specimens and negative for the others (data not shown). Therefore, the primers and probes were specific for MB HPV16 DNA measurement. In order to quantify the amount of cellular DNA in the sample, MB β-actin DNA copy numbers were measured using TaqMan primers and a probe (catalog no. 401846; Applied Biosystems) and the median MB β-actin copy number from 15 MB HPV16 DNA-positive patient samples was used to normalize the HPV16 DNA copy numbers for those 15 patients.

QRT-PCR was performed using a reaction volume of 25 μl and TaqMan Universal PCR Master Mix (catalog no. 4304437; Applied Biosystems), and 100 ng in 2.5 μl of extracted genomic DNA was used for each reaction. The standard protocol for the ABI Prism 7500 sequence detector (Applied Biosystems) was used. Absolute MB HPV16 and MB β-actin copy numbers for each patient sample were obtained using standard curves prepared by amplification of the respective plasmid-cloned HPV16 DNA and β-actin DNA at known input concentrations ranging from 0 to 1,000,000 copy numbers. Plasmids containing HPV16 and β-actin sequences were prepared by cloning their PCR products separately into pCR2.1 vector (TOPO TA cloning kit; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and plasmids with integrated sequences were purified using a QIAprep Spin Miniprep kit (Qiagen GmbH, D-40724 Hilden, Germany) according to the manufacturer's instructions. Each batch of amplifications included multiple positive and negative controls. Duplicate tests were performed, and averages were calculated for each sample.

**Statistical analysis.** Copy numbers were $\log_{10}$-transformed and summarized in scattered plot graphs. The nonparametric Spearman rank correlation test was used to measure the correlation between normalized MB HPV16 DNA copy numbers and stages of CIN.

## RESULTS

**MB HPV DNA detection and genotyping.** MB HPV DNA was detected in (i) 82.6% (194/235) of patients with CIN or CAC; (ii) 22.2% (24/108) of patients with HGCIN after LEEP treatment; and (iii) 0.0% (0/62) of patient with LGCIN or CAC with complete remission. Stratifying the patients into different histological stages, MB HPV DNA was detected in 100% (48/48) of patients with CIN 3, 100% (60/60) of patients with CIN 2, 72.4% (42/58) of patients with CIN 1, and 63.8% (44/69) of patients with CAC. Overall, MB HPV DNA was found in 100% (108/108) of patients with HGCIN and 67.7% (86/127) of patients with LGCIN or CAC. A single HPV risk type was detected in MB from 69.4% (75/108) of HPV DNA-positive HGCIN patients and 80.2% (69/86) of HPV DNA-positive LGCIN or CAC patients, whereas double HPV risk types were detected in MB from 30.6% (33/108) of HPV DNA-positive HGCIN patients and 19.8% (17/86) of HPV DNA-positive LGCIN or CAC patients. Moreover, MB high-risk (HR) HPV DNA was detected in 89.8% (97/108) of HPV DNA-positive HGCIN patients and 26.7% (23/86) of HPV DNA-positive LGCIN or CAC patients. On the other hand, MB HPV DNA was detected in 8.1% (26/323) of samples from ANS; among these, 30.8% (8/26) MB HPV DNA-positive ANS had HR HPV and 69.2% (18/26) MB HPV DNA-positive ANS had low-risk (LR) HPV. Histology confirmed that 2 MB HR HPV DNA-positive ANS had CIN 1 and that 2 MB LR HPV DNA-positive ANS had CAC, whereas the remaining 22 MB HPV DNA-positive ANS (6 HR and 16 LR) had not yet been found to have CIN or CAC in their cervical smears. Furthermore, 297 ANS without MB HPV DNA were all found to have normal cytology results. Therefore, the status of those 4 ANS who had CIN 1 or CAC was changed from ANS to the patient category; the adjusted results showing the prevalence of HPV risk types detected in MB of patients with HGCIN, LGCIN, or CAC and and in MB of ANS are shown in Table 1. Overall, the sensitivity, specificity, and positive and negative predictive values for MB HPV DNA in detection of CIN and CAC were 82.8% (198/239), 93.1% (297/319), and 90.0% (198/220) and 87.9% (297/338), respectively. The numbers of various HPV genotypes detected in MB from HPV DNA-positive subjects before treatment or remission are shown in Fig. 1; the percentages of MB HPV16, -18, -31, and -33 among all HPV-positive subjects were 5.6% (15/270), 7.0% (19/270), 3.0% (8/270), and 5.9% (16/270), respectively. All results from duplicate experiments were consistent, and MB β-globin DNA was detected in all patient samples.

**MB HPV16 DNA quantitation.** Test results for detection of MB HPV16 DNA were confirmed by direct sequencing to be positive for 9 patients with CIN 3, 4 patients with CIN 2, and

TABLE 1. Prevalence of HPV risk types detected in the menstrual blood of patients with HGCIN, LGCIN, or CAC and of ANS

| Diagnosis | HPV DNA (%) | % single HPV risk type (no. of samples with positive result/total no. of samples) | | | % double HPV risk type (no. of samples with positive result/total no. of samples) | | | |
|---|---|---|---|---|---|---|---|---|
| | | HR | LR | Unknown risk | HR/HR | HR/LR | LR/LR | LR/unknown risk |
| CIN 3 | 100.0 (48/48) | 56.3 (27/48) | 0.0 | 0.0 | 27.1 (13/48) | 14.5 (7/48) | 2.1 (1/48) | 0.0 |
| CIN 2 | 100.0 (60/60) | 66.7 (40/60) | 13.3 (8/60) | 0.0 | 10.0 (6/60) | 6.7 (4/60) | 3.3 (2/60) | 0.0 |
| CIN 1 | 73.3 (44/60) | 23.3 (14/60) | 31.7 (19/60) | 0.0 | 0.0 | 1.7 (1/60) | 13.3 (8/60) | 3.3 (2/60) |
| CAC | 64.8 (46/71) | 14.1 (10/71) | 42.3 (30/71) | 0.0 | 0.0 | 0.0 | 5.6 (4/71) | 2.8 (2/71) |
| ANS | 6.9 (22/319) | 1.9 (6/319) | 5.0 (16/319) | 0.0 | | | | |
| CIN 3 or 2 after LEEP treatment | 22.2 | | | | | | | |
| CIN 1 or CAC after recovery | 0.0 | | | | | | | |

2 patients with CIN 1. QRT-PCR showed that test results for detection of MB HPV16 DNA (Fig. 2) and MB β-actin DNA (range of copy numbers, 16,995 to 135, 632; median copy number, 78,988) were positive for all 15 patients with various copy numbers. Detailed analysis showed that the normalized MB HPV16 DNA copy numbers in samples from patients with CIN 1 to CIN 3 were significantly increased ($P < 0.005$; Spearman rank correlation test).

## DISCUSSION

MB provides a rich and stable source of materials for detection of HPV DNA. Compared to the Pap test, collection of MB is completely noninvasive and women do not need to visit outpatient clinics for sample collection using a cytobrush; thus, both pain and embarrassment can be avoided.

The success in detecting and typing MB HPV DNA in samples from 82.8% of patients with CIN or CAC, 22.2% of patients with HGCIN after LEEP treatment, and 0.0% of patient with LGCIN or CAC with complete remission justifies a long-term follow-up for patients with detectable MB HPV DNA in order to examine the prognostic significance of the test. Moreover, the excellent diagnostic accuracy of this noninvasive test proves that it has a high potential to screen ANS and for triage evaluations for those with MB HR HPV DNA for the Pap test. Hence, our work has potential clinical applications in 3 scenarios: (i) routine screening for women where the necessary infrastructures for cytology screening are not available (3); (ii) routine screening for women who are reluctant to consult doctors due to pain or embarrassment even when symptoms appear; (iii) more frequent monitoring of patients than with the Pap test due to the noninvasive nature of the collection method.

To our knowledge, this study is the first to use MB samples from patients with CIN or CAC for HPV DNA detection. Although inserting a tampon inside the vagina has been used as a method of self-collection of samples for HPV detection, resulting in a range of sensitivity from 67% to 94% (8), this

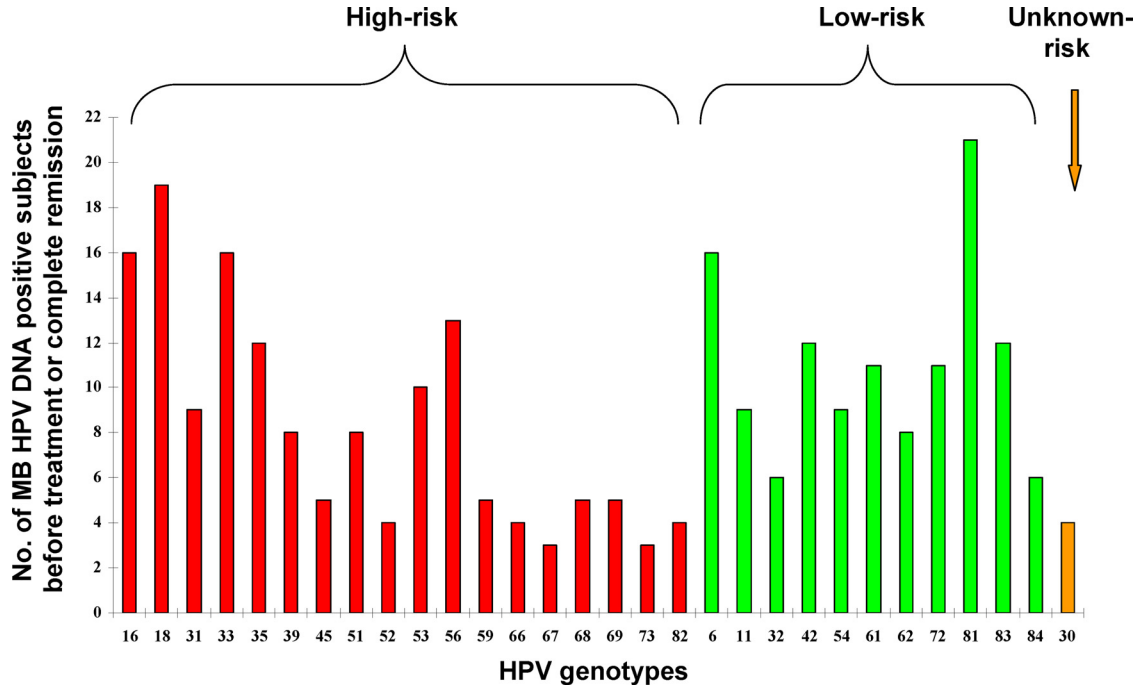


FIG. 1. Numbers of various HPV genotypes detected in MB HPV DNA-positive subjects before treatment or complete remission.

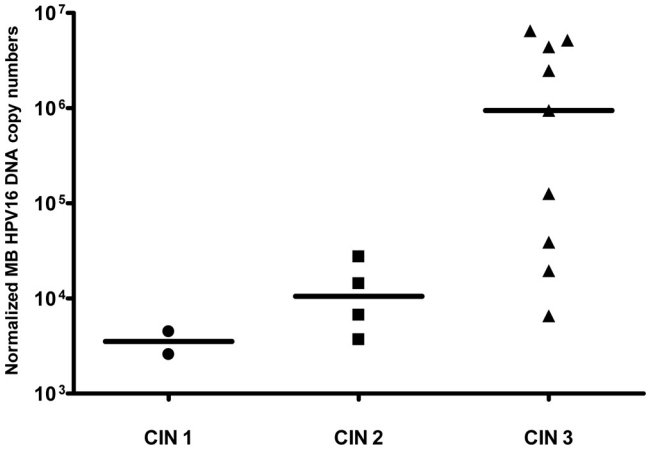


FIG. 2. Normalized MB HPV DNA16 copy numbers in MB samples from 15 HPV16 DNA-positive patients with CIN at different stages. The median is indicated by a black horizontal line.

method creates discomfort for women, and the detection rate varies according to the duration of the application and the depth the tampon reaches inside the vagina (4). The use of MB can circumvent these limitations, as MB with HPV DNA is released and collected in the sanitary napkin during menstruation. Moreover, self-collected cells from tampons or vaginal swabs require liquid-based storage and transport media, which are inflammable and hazardous (4), whereas MB collected in sanitary napkins can be stored inside a zip-lock bag for transportation. Another study by Tong et al. detected HPV DNA in 100% (17/17) of patients with CIN or CAC by the use of vaginal discharge (VD) collected on sanitary napkins (16). In comparisons of HPV DNA detection using MB to HPV DNA detection using VD, MB has an advantage, because MB can be collected monthly from women before menopause whereas VD cannot be collected regularly.

In our patient cohort, the sensitivity of the use of HR HPV DNA in MB samples to detect HGCIN was 89.8%, which is much higher than that obtained using self-collected vaginal swabs (66.1%) (18) and is comparable to that obtained using cervical tissue (94.6%) (11). A major limitation of our study was that the use of direct sequencing to genotype the HPV DNA is less sensitive for detection of multiple HPV infections than advanced methods such as the line probe genotyping assay (1), because only 2 HPV types can be detected. In order to validate our results obtained using MB samples with double HPV risk types, all MB samples with double HPV risk types were selected for HPV DNA detection again, using another pair of established GP5+ and GP6+ primers followed by HPV genotype detection in all HPV DNA-positive samples by direct sequencing using the GP5+ primer (9). Our results showed that all MB sample double HPV risk types detected using MY11 for direct sequencing were fully matched to those detected using GP5+ primers (data not shown); therefore, the detection of double HPV risk types in MB samples was accurate. Another cohort of patients with CIN is now being recruited for studies designed to compare the sensitivity of HR HPV detection using this approach to that of the conventional method using Digene Hybrid Capture technology in ThinPrep samples. Furthermore, QRT-PCR showed that the normalized MB HPV16 DNA copy numbers may reflect the severity of CIN. However, a few patients with CIN 3 had relatively low HPV16 DNA copy numbers, which may indicate a weak predictive value (Fig. 2). As only 15 HPV16 DNA-positive specimens were detected in this study, we need to validate our results using a larger cohort of HPV16 DNA-positive specimens in the future. On the other hand, the broad range of MB β-actin DNA copy numbers obtained for those 15 MB HPV16 DNA-positive patients suggests that normalization of MB HPV16 DNA results is essential and that the broad range of MB β-actin DNA copy numbers may have 2 possible explanations: (i) various degrees of cellular degradation were found in those MB specimens; and (ii) β-actin DNA may not be an appropriate housekeeping gene in MB specimens. Therefore, standardization of MB collection and delivery procedures is necessary to prevent inconsistencies in DNA quality that may lead to inaccurate HPV16 DNA quantitation.

Despite this, our finding is still important because it shows that the normalized MB HPV16 DNA load may be useful for the follow-up of CIN patients in cases of relapse after treatment or progression of CIN. According to previous reports from Europe and HKSAR, HPV16 infections occur more frequently in younger people, ranging from early 20s to 29 years of age, and uncommon HR or probable HR HPV types such as HPV35, -45, -51, -53, -58, -70, and -73 occur more frequently with increasing age (2, 6, 14). Therefore, the relatively low prevalence of HPV16 DNA-positive patients in our study can be explained as follows: (i) the age group of our patient cohort ranged from 17 to 54 years, with a mean age of 37.3, and the risk of the presence of infecting HPV16 in this patient group was therefore relatively lower than the risk would have been for a cohort with a younger age range (2, 6, 14); (ii) members of the patient cohort in our study first experienced sexual intercourse at a mean age of 29, as disclosed in a questionnaire designed by Wong et al. for subjects participating in our study, and the risk of the presence of infecting HPV16 in this patient group is relatively lower than the likely risk for subjects with a younger age of earlier sexual experience; and (iii) the cohort in our study did not include patients with cervical cancer, who exhibit a high prevalence of HPV16 infection (6, 14).

Currently, a multicenter study designed to use MB HPV DNA in samples from patients with CIN or CAC in various age and sexual experience groups is in progress in order to validate our results, and 6 MB HR HPV DNA-positive ANS with normal cytology are being followed up to examine whether CIN or CAC will develop in the future. Our results are still preliminary, and three important issues have to be resolved before attempting to use MB HPV DNA detection and genotyping in a large-scale screening: (i) exploration of the proper way to store the samples and transport to the collection centers in order to standardize the collection method so that sample degradation can be kept to a minimum and to ensure that the transportation process is safe, because MB collected in a sanitary napkin is usually treated as garbage disposed in a litter bin; (ii) use of high-throughput processing equipment such as an automatic cutting machine to cut MB specimens and of an automatic extraction workstation to streamline the workflow for DNA extraction; and (iii) optimization of MB specimens for HPV genotyping using methods other than direct sequencing such as the line probe genotyping assay to increase detec-

tion sensitivity and for easy reading of results, especially in cases of multiple HPV infections.

In summary, our findings not only demonstrate that MB is an appropriate source for noninvasive detection for HPV DNA but also open up the exciting possibility of the use of MB to detect other DNA aberrations such as mutation or methylation in PCD. We strongly hope that a noninvasive MB HPV DNA test with higher sensitivity and specificity than the conventional Pap test can be developed, with the ultimate aim of reducing the incidence of CC with a cost-effective and readily available test.

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